

CHEMICAL DEFENSES OF THE FIRE ANT,
SOLENOPSIS INVICTA BUREN, AGAINST INFECTION BY
THE FUNGUS, BEAUVERIA BASSIANA (BALSAMO) VUILL.

By

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Abstract of Dissertation Presented to the Graduate School
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CHEMICAL DEFENSES OF THE FIRE ANT,
SOLENOPSIS INVICTA BUREN, AGAINST INFECTION BY
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Solenopsis invicta venom alkaloids and cuticular hydrocarbons were evaluated for antimycotic activity against Beauveria bassiana. Cuticular hydrocarbons were non-inhibitory in both liquid and solid media assays. Hydrocarbons supported conidial germination and subsequent fungal development when present as the sole carbon source on agarose. Alkaloid concentrations $> 20 \text{ ug per cm}^2$, caused $> 80\%$ inhibition of conidial germination in both solid and liquid media assays for 24 hours post-treatment. Percent germination increased within 48 hours, indicating a fungistatic effect of venom alkaloids. The developmental sequence of the fungus also was modified by the alkaloids, resulting in induction of the hyphal body phase. In solid media assays, scanning electron microscopy of agar surfaces

24 hours post-treatment revealed that the fungus occasionally germinated perpendicular to venom-treated agar surface.

Release of venom alkaloids into sand by 50 fire ant workers was quantified by gas chromatographic analysis of methanol extracts of sand taken from laboratory assay units. Alkaloid levels were significantly higher in B. bassiana inoculated sand than in noninoculated sand at 7 and 14 days post-treatment. However, alkaloid levels in the sand were below those defined as fungistatic in vitro. Infection of fire ants in inoculated sand was less than 40%.

Both inoculated and noninoculated sand from fire ant assay units used in the alkaloid release studies caused almost 90% mortality of Artipus floridanus larvae in bioassays. However, infection of the larvae by B. bassiana was expressed in only 20% of the cadavers from inoculated assay unit sand compared to over 80% infection of A. floridanus in inoculated non-ant sand. Sterilizing assay unit sand removed the fungicidal and insecticidal activities of the sand. Venom alkaloid spiked sand was neither insecticidal to A. floridanus larvae nor inhibitory to the infection of the larvae by B. bassiana.

SECTION 1 INTRODUCTION

The entomogenous fungus Beauveria bassiana (Balsamo) Vuillimen is currently being tested in the laboratory and field as a potential microbial insecticide in several countries including the United States. The majority of commercial production and application of the fungus has occurred in eastern Europe and the Soviet Union, primarily for the control of the Colorado potato beetle, Leptinotarsa decemlineata (Say), and the codling moth, Cydia pomonella (L.) (reviewed by McCoy et al. 1988). The People's Republic of China produces enough B. bassiana to treat > 1 million hectares of pine forest annually for control of Dendrolimus spp. (Roberts 1989). Commercialization of the fungus is expected in France where it will be used against the European corn borer, Ostrinia nubilalis (Riba 1984, McCoy 1989). Considerable effort has been made in the United States to develop specific isolates of B. bassiana for control of various insects including the Colorado potato beetle (Roberts et al. 1981; Watt and Lebrun 1984, Campbell et al. 1985; Anderson et al. 1988; Gaugler et al. 1989), different citrus root weevils (reviewed by McCoy 1989), and

the pecan weevil, Curculio caryae (Horn) (Gottwald and Tedders 1983; 1984).

Fungal conidia have been applied to the soil for controlling several soil-inhabiting insect pests. For example, B. bassiana applied to the soil in potato fields reduced emergence of the first- and second-generation Colorado potato beetle adults by 74 and 77%, respectively, over untreated plots (Watt and Lebrun 1984). Likewise, McCoy et al. (1985) report >70% infection of the little leaf notcher, Artipus floridanus, larvae following soil application of B. bassiana conidia.

Since it's importation from South America, the fire ant, Solenopsis invicta Buren, has attained introduced pest status in the southeastern United States (Lofgren et al. 1975). Presently, B. bassiana is being evaluated as a potential microbial control agent of this ant (Alves et al. 1988, Dr. J.L. Stimac personal communication).

Fungal pathogens frequently cause epizootics in insect populations (reviewed by Carruthers and Soper 1987; Carruthers and Hural 1989). Epizootics of B. bassiana have been reported for many insects as a result of contact with a natural reservoir of conidia in the soil (Ferron 1981). Disease incidence increased in Egyptian alfalfa weevil populations in southern California alfalfa fields after crop harvest or when rainfall knocked the insects to the soil (Johnson et al. 1984). Likewise, Schvester (1957) reported

that entire populations of larval Scolytus rugulosus Muller are killed by a fungus presumed to be B. bassiana during rainy seasons in France (in Doberski and Tribe 1980).

It is generally accepted that high host population density contributes to the development of epizootics (Carruthers and Soper 1987; Keller and Zimmermann 1989). Populations of the citrus rust mite, Phyllocoptura oleivora (Ashmead) are regulated by bi-annual epizootics of the fungus, Hirsutella thompsonii Fisher, only at high mite densities after crop damage (Muma 1955; McCoy et al. 1976). Likewise, populations of the cockchafer, Melolontha sp., are controlled by epizootics of B. brogniartii (Saccardo) Petch (in Keller and Zimmermann 1989). Entomophthorales fungi frequently caused epizootics in aphid (Soper 1981), leafhopper (Soper 1985) and alfalfa weevil (Johnson et al. 1984) populations. Seasonal Nomuraea rileyi epizootics in populations of the velvet bean caterpillar, Anticarsia gemmatilis, have been observed in soybeans in the southeastern United States (Kish and Allen 1978).

Extensive surveys of fire ant colonies in both the United States and South America have failed to identify B. bassiana as an important pathogen of S. invicta populations (reviewed by Jouvenaz 1983; Jouvenaz 1986) although the fungus is present in the mound environment (Alves et al. 1988). Carruthers and Hural (1989) reported that workers in Florida have "circumstantial evidence" that B. bassiana

epizootics may cause as much as 50% reductions in some fire ant populations in Brazil.

Fire ants exist under conditions that under many circumstances would be considered suitable for the development of fungal epizootics. First, B. *bassiana* is present in mound soil at concentrations of 10^2 to 10^6 conidia per gram of soil and infectious to fire ant workers (Dr. J.L. Stimac, personal communication). Second, fire ant colonies typically contain over 50,000 workers (Wojcik 1986) which interact intimately during grooming and feeding, increasing the possibility of contact-mediated disease transmission. Finally, environmental conditions in the mound are optimal for fungal growth and development with a monthly mean soil temperature in Mato Grosso, Brazil, of 24 to 27.7°C (Banks et al. 1985) and generally, relative humidity of soil exceeds 99% (Keller and Zimmermann 1989).

Given that the soil serves as a natural reservoir of fungal conidia and excellent medium for dissemination of conidia (Ferron 1981; Carruthers and Soper 1987), such a high concentration of insects under optimal conditions for fungal development with little disease incidence suggests that mechanisms are in place and functioning to prevent B. *bassiana* disease development.

Many factors can limit fungal infection and subsequent epizootics. Keller and Zimmermann (1989) recently reviewed the factors influencing fungal epizootics in soil. Briefly,

abiotic factors such as temperature and humidity are important regulatory factors in disease development. Also, biotic factors, such as microorganisms, arthropods and their associated metabolites can inhibit soil-borne fungi. Secondary plant compounds can also function as inhibitors of microorganisms in the soil. Other factors, such as, conidial population density, viability, virulence, and spatial distribution in the environment can also affect fungal epizootiology (Carruthers and Hural 1989).

Several potential disease-limiting mechanisms are found in the fire ant mound environment. First, fire ants, like other social insects, maintain extremely clean mounds by removing debris, including ant cadavers, within 1 day of deposition (Wilson 1971). Entomopathogenic fungi typically require 2-3 days after host death before sporulation occurs. Fungus-infected cadavers are removed prior to sporulation, thereby limiting the dissemination of conidia within the mound. In preliminary assays where laboratory fire ant colonies were inoculated with B. bassiana, ant cadavers were removed by ants to one corner of the assay unit prior to sporulation of the fungus. Following sporulation of the fungus, the sporulating cadavers were covered with sand by the fire ant workers, presumably to limit dispersion of conidia.

Second, antimicrobial chemicals are often associated with ants. For example, metapleural gland secretions from

the Australian ant, Myrmecia nigriscapa Roger, significantly reduce in vitro conidial germination of several fungi, including B. bassiana (Beattie et al. 1985; 1986). Metapleural gland secretions of fire ants are not antimicrobial (in Obin and Vander Meer 1985) but function as territorial pheromones in Solenopsis geminata (Jaffe and Puche 1984). However, fire ant venom alkaloids have significant antimicrobial activity in in vitro laboratory assays (Blum et al. 1958; Jouvenaz et al. 1972; Blum 1988) and are thought to have evolved to function, at least partially, as disinfectants of the ant cuticle and mound soil (Cole 1975; Obin and Vander Meer 1985; Blum 1988).

Large amounts of venom alkaloids are available in each nest since each worker venom sac has ca. 10-15 ug of alkaloids (R.K. Vander Meer, personal communication). The venom is apparently dispersed into the mound environment via the rapid vibration of the sting, gaster flagging, during which small quantities of venom are released (Obin and Vander Meer 1985). The presence of these antimicrobial alkaloids in the mound and on the ants makes contact between fungal spores and alkaloids possible. The interaction of alkaloids and fungal conidia could inhibit disease development.

Finally, the fungal infection of insects is initiated on the insect cuticle with conidial attachment, germination, and germ tube penetration of the exoskeleton. Inhibition of

any of these processes by cuticular components could prevent the successful infection of the host.

Cuticular components of several insects have proven to be either stimulatory (Woods and Grula 1984; Boucias and Pendland 1984; Boucias and Latge 1988; Kerwin 1984) or inhibitory (Koidsumi 1957; Evalakova and Chekhourina 1962; Smith and Grula 1982) to the germination of different fungi. Cuticular hydrocarbons constitute over 70% of the total lipids on the fire ant cuticle (Lok et al. 1975). The effect of fire ant cuticular hydrocarbons on fungi has not been previously reported.

The action of fire ant chemicals on B. bassiana must be known to properly evaluate the potential of this fungus as a microbial control agent since the presence of inhibitors in the mound could limit efficacy of the fungus. The results should have broad applicability to other potential fire ant microbial control agents since the antimicrobial activity of the venom appears to affect several pathogens (Blum 1988). Therefore, the main purpose of the studies reported herein was to quantify the inhibitory effects of different chemicals present in S. invicta nests against B. bassiana and other entomogenous fungi and measure the release of venom alkaloids by fire ants in response to fungal challenge. Specific research objectives were as follows:

1. Quantify the fungistatic effects of S. invicta cuticular hydrocarbons and venom alkaloids on entomopathogenic fungi.

2. Determine the effect of venom alkaloids on the morphological development of B. bassiana.
3. Quantify the behavioral response of S. invicta to nest sand inoculation with B. bassiana.
4. Quantify mycosis of weevil larvae by B. bassiana in sand containing venom alkaloids.
5. Determine the fungistatic and insecticidal effects of sterile and nonsterile fire ant sand on B. bassiana conidia and weevil larvae, respectively.

SECTION 2 MATERIALS AND METHODS

Culturing Fungi and Determining Conidial Viability

A monospore isolate of Beauveria bassiana (strain AF-4), isolated from Artipus floridanus by Dr. C.W. McCoy and maintained at -70°C in the culture collection of the Citrus Research and Education Center, University of Florida was used for most experimentation. Monospore isolates of Metarhizium anisopliae (Metsch.) Sorokin from the mole cricket (Scapteriscus vicinus) and Paecilomyces fumoso-roseus (Wize) Brown and Smith from an unidentified host insect were obtained from the fungal culture collection (maintained at -70°) of the Entomology and Nematology Department, University of Florida. A monospore isolate of B. bassiana (strain 447) isolated from S. invicta was obtained from Dr. Jerry L. Stimac (Entomology and Nematology Dept., University of Florida, Gainesville). Pure cultures of the fungi were grown on Sabouraud's dextrose agar (SDA) in Petri dishes (15mm x 100mm) at 27°C. After 10 days, conidia were harvested by scraping the plates with a sterile microscope slide. Conidia were then placed in 10 ml sterile deionized water (SDW), vortexed 1 min, and washed twice in SDW using centrifugation (5000xG, 5 min) to remove agar residue.

Concentration of the final conidial suspension was determined by hemacytometer counts. Conidial suspensions were then stored at -20°C . All experiments were conducted with aliquots of conidia harvested and frozen by this method. After an aliquot was thawed and used for experimentation, the unused portion was discarded to reduce the possibility of damage to conidia by repeated thawing.

Viability of conidia was evaluated at the beginning of each experiment by inoculating 100 μl of Sabouraud's dextrose broth (SDB) with 100 μl of the conidial preparation and incubating the broth for 24 hours at 27°C . Conidial germination, defined by the presence of a germ tube, was quantified by counting ca. 100 spores in each of 4 replicates.

Field Collection and Maintenance of *Solenopsis invicta* Laboratory Colonies

Fire ants used for venom collection and sand assay experimentation were collected and maintained according to the procedures of Banks et al. (1981). Ants were collected by shoveling the upper portion of the nest tumulus into a bucket with the inner rim coated with Fluon^R (ICI United States, Wilmington, Del.) to prevent escape. Ants were returned to the laboratory and separated from the soil using the flotation method of Jouvenaz et al. (1977). Ants were recovered from the water surface using a large spoon, then

transferred to a Fluon-coated plastic tray (44 cm x 56 cm x 7 cm, Panel Controls, Detroit, Mich.) containing a Williams' nest cell (Banks et al. 1981). Water was added periodically to the chamber by syringe injection and water-filled test tubes plugged with absorbent cotton were placed in each tray. Honey agar (10 g honey and 1.5 g agar in 100 ml water), a boiled egg, and various lepidopteran and coleopteran larvae were provided weekly as food.

Rearing Procedures for *Artipus floridanus* Larvae

Larvae of the little leaf notcher, *Artipus floridanus* Horn (Coleoptera: Curculionidae), were used in sand bioassay experimentation to measure the effect of mortality factors in both sterile and nonsterile sand. Larvae were reared on an artificial diet at the University of Florida, Citrus Research and Education Center, Lake Alfred, Fla., according to the methodology described by McCoy et al. (1985). Larvae (45-days old) of uniform size and weight were used in all bioassays involving this insect.

Extraction and Purification of Cuticular Hydrocarbons

Crude cuticular extract from ca. 1 kg of whole *S. invicta* workers was provided by Dr. Robert K. Vander Meer (USDA/ARS, Insects Affecting Man and Animals Research Laboratory, Gainesville, Fla.) as a source of hydrocarbons. The extract was prepared by soaking the ants for an undetermined length of time in hexane and the solvent removed by vacuum evaporation leaving a residue of 2.7 g.

Qualitative gas chromatographic (GC) analysis indicated cuticular hydrocarbons and venom alkaloids as the major constituents of the extract in a 2.8 to 1 ratio, respectively.

Cuticular hydrocarbons in the crude extract were isolated by silicic acid column chromatography using BIO-SIL HA (-325 mesh, Bio Rad, Richmond, CA). The column bed size was 6.3 cm dia. X 7.5 cm. Approximately 1 g of the crude extract was applied to the surface of the column and eluted with hexane (Christie 1973). The hexane eluate was collected in 50 ml aliquots which were monitored for the presence of hydrocarbons by thin layer chromatography (TLC). The TLC plates were visualized with iodine vapor. All positive fractions were confirmed qualitatively by GC (Figure 1). All cuticular hydrocarbon fractions were combined, concentrated by vacuum evaporation, and quantified by GC analysis (see pg 14).

Isolation of Venom Alkaloids from Fire Ants

Venom alkaloids used in all experiments were obtained from laboratory-colony fire ant workers following extirpation of the venom sacs by dissection. Large worker ants were killed in either methanol or hexane and the venom sac removed by tearing the last 2 dorsal abdominal sclerites, grasping the sting apparatus with forceps and pulling the venom pouch free of the abdomen. The venom sac was separated from the sting by dissection and placed in a

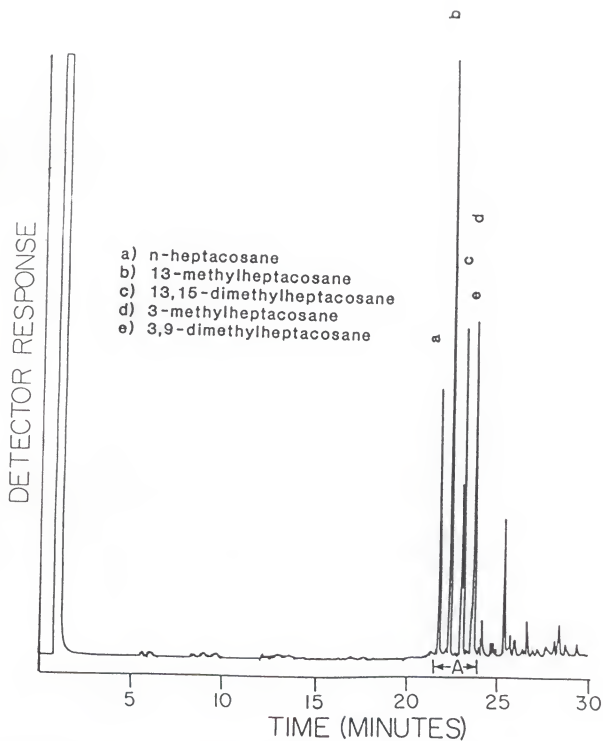


Figure 1. Chromatographic patterns of 5 major hydrocarbons of *S. invicta* worker cuticular extract.

vial containing either methanol or hexane and the venom released by crushing the sac with forceps. Concentration of venom alkaloids in the solution was determined by GC analysis (Figure 2).

Quantification of Fire Ant Compounds
Via Gas Chromatography

Venom alkaloids and cuticular hydrocarbons were separated using either a Varian 3700 Gas Chromatograph (Varian Associates, Sunnyvale, CA) equipped with a 30 m DB-1 column or a Tracor 540 Gas Chromatograph (Tracor Instruments Austin, Austin, TX) equipped with a 15 m DB-5 column. Both GC's were equipped with flame ionization detectors. The ovens were programmed from 150°C to 275°C-285°C at 5°C per min and data collected using either a Varian Vista 401 Data Processor or a Perkin-Elmer LCI-100 Integrator. Six or ten microliters of a 0.1% hexane solution of either tetracosane or docosane were added as internal standards prior to analysis. Sample quantification was determined by dividing the total alkaloid or hydrocarbon peak area by the peak area of the internal standard and multiplying by the amount of standard (in ug) added to the sample.

Effect of Fire Ant Compounds on Conidial Viability
in Solid and Liquid Culture

Cuticular Hydrocarbons

The effect of cuticular hydrocarbons on the germination and development of B. bassiana conidia on solid substrates

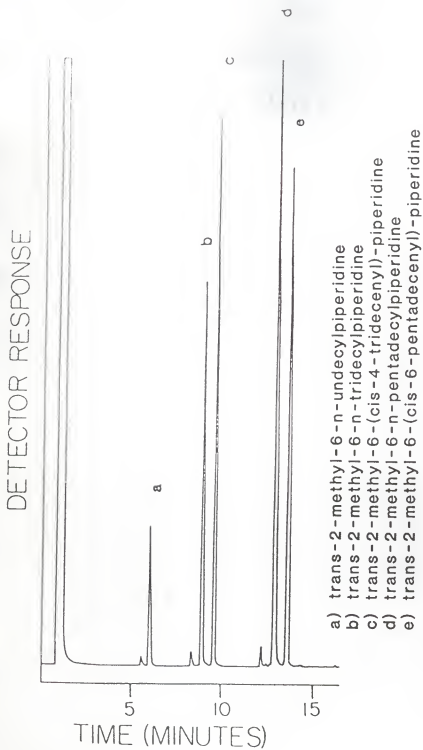


Figure 2. Chromatographic patterns of 5 major alkaloids of S. invicta worker venom.

was quantified. Tenfold serial hexane dilutions of cuticular hydrocarbons were filter sterilized using an HPLC solvent syringe filter (0.45u, MSI Cameo). Five microliters of each dilution were spot-plated onto both Millicu (MC) agar (0.36g KH_2PO_4 , 1.05g Na_2HPO_4 , 0.6g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0g KCl, 10g glucose, 0.7g NH_4NO_3 , 5.0g yeast extract, 20g agar per l) and 1.5% agarose (Bio-Gel, Richmond, CA) prepared with double distilled deionized water. Five concentrations of cuticular hydrocarbons (0, 0.25, 2.5, 25.2 and 251.6 $\mu\text{g}/\text{cm}^2$) were tested. Concentration per unit area was calculated based on the area covered by a 5 μl droplet of hexane on the agar surface (0.636 cm^2). The hexane was evaporated from the agar surface and the plates sprayed with an aqueous suspension containing 5×10^6 viable B. bassiana conidia per ml. Plates were incubated at 27°C. Conidial germination was quantified after 24 and 48 hours by counting ca. 100 spores per replicate for each treatment concentration and identifying the presence or absence of a germ tube using phase-contrast microscopy (400x). Each concentration was replicated 2 times.

The effect of cuticular hydrocarbons on the germination and growth of B. bassiana in liquid culture was quantified using the previously described solid media methodology with the following exception. Five microliter aliquots of the cuticular hydrocarbon dilutions were spot plated in individual wells of a 96-well microtiter plate. Five

concentrations of cuticular hydrocarbons (0.0, 0.24, 2.4, 24.1 and 241 ug per cm²) were evaluated. Concentration per unit area was based on the surface area of the bottom of a well (0.332 cm²). B. bassiana conidia at a concentration of 5×10^6 conidia per ml were suspended in either Sabouraud's dextrose broth (SDB) or SDW and added to the treated wells in 100 ul aliquots. Plates were incubated at 27°C and observed after 24 and 48 hours for conidial germination by inverted phase microscopy (400x). Each rate was replicated 4 times.

Venom Alkaloids

The effect of selected concentrations of venom alkaloids on in vitro germination and development of B. bassiana (AF-4) on SDA and agarose media was tested using a modification of the previously described protocol (page 16). A template, constructed by cutting the bottom from a 96-well microtiter plate, was surface sterilized with ethanol and used to hold 10 ul of venom alkaloids on solid substrates within a 0.332 cm² area during solvent evaporation. Six concentrations (0, 3.8, 7.6, 15.1, 30.1, and 60.2 ug per cm²) were evaluated. Each concentration was replicated 4 times.

The effect of different concentrations of venom alkaloids on in vitro germination and development of B. bassiana in SDB was tested using previously described microtiter well coating protocol (page 16). Seventy-two

venom alkaloid concentrations ranging from 0 to 39.6 ug per cm^2 were tested and germination quantified for 105 observations. Inhibitory activity of venom alkaloids to fungal conidia was compared at 24 and 48 hours. Conidial germination was quantified as described previously with one exception. At some concentrations, hyphal bodies were induced by the venom alkaloids and were excluded from germination counts. Only conidia with germ tubes were counted as germinated.

The effect of different concentrations of venom alkaloids on in vitro conidial germination of other B. bassiana isolates, M. anisopliae and P. fumosoroseus in liquid culture was compared using the described protocol above. Eight concentrations of venom alkaloids ranging from 0.0 ug to 29.7 ug per cm^2 were tested. Each concentration was replicated 4 times.

Effect of Liquid Medium on Alkaloid Activity

Coated Well Method

Previous experiments indicated that the inhibitory effect of venom alkaloids on B. bassiana germination in coated well assays was overcome by the fungus within 48 hours after treatment. The possibility of fungal recovery due to dilution of the venom alkaloids by the broth was tested using the coated well assay protocol with one exception. Germination of conidia added to wells at 0 hour was compared to germination of conidia added 24 or 48 hours

after the addition of the alkaloids to wells. Seven concentrations ranging from 0 to 39.2 ug alkaloids per cm^2 were tested. Each concentration was replicated 3 times.

Broth Incorporation Method

The effect of different concentrations of venom alkaloids solubilized in liquid medium on in vitro germination and development of B. bassiana was tested. Venom alkaloids were collected using the previously described dissection procedure, then solubilized in absolute ethanol, and finally quantified by GC. The ethanol solution was then added to SDB to a final concentration of 1% (v/v) and serially diluted by 2-fold with 100 ul of SDB in a 96 well microtiter plate. B. bassiana conidia were introduced at 0, 24, and 48 hours, by adding 10 ul of a conidial suspension containing 5×10^5 spores per ml. Germination and hyphal development of the fungus for each inoculation time were observed after 24 and 48 hours incubation at 27°C. Seven venom concentrations ranging from 0 ng per ul to 62 ng per ul SDB were tested. Each concentration was replicated 3 times. **Note:** The concentrations tested were comparable to those used in the coated well assay described on page 18. The concentrations in that experiment were calculated based on the surface area of the well bottom. If calculated based on the amount of venom added per 100 ul of SDB, venom concentrations ranged from 0 to 118 ng per ul.

Qualitative Examination of Conidial Germination Using Scanning Electron Microscopy

Conidia exposed to 6 concentrations of venom alkaloids (0, 1.6, 2.5, 5, 10, and 20 ug alkaloid per cm²) applied to SDA were examined using scanning electron microscopy. At 24, 48, and 72 hours post-treatment, agar plugs from treated areas were fixed in osmium tetroxide vapors for 96 hours, then desiccated over silica gel for 96 hours. Samples were gold coated and examined using a Hitachi S530 scanning electron microscope operating at 20 KV.

Extraction of Venom Alkaloids from Sand

Venom alkaloids collected from 10 large fire ant workers in 0.5 ml methanol were quantified by GC analysis and then added to 10 g sand (Feldspar Corp., Edgar, FL) contained in a 50 ml glass beaker. The vial containing the venom was rinsed 3 times with methanol and each rinse added to the sand. After the methanol solution of venom was thoroughly mixed with the sand, the mixture was oven dried at 40°C for 30 min. The dried sand was placed in a chromatography column (30 cm x 8 cm diam) with glass wool blocking the exit hole. Alkaloids were extracted from the sand column by eluting 5 times with 20 ml methanol aliquots. The total eluent (100 ml) was collected in a 250 ml round bottom flask and the solvent vacuum evaporated at 37°C and 25 PSI. The flask was rinsed three times with methanol (5 ml each). The combined rinses were evaporated under a stream of

N₂ at 37°C. Any remaining residue in the 20 ml vial was rinsed 3 times to a total volume of ca. 1.5 mls, collected in a 2 ml screw cap vial, and concentrated by N₂-evaporation to 500 ul. Docosane was then added as an internal standard and the venom alkaloids in the extract were quantified by GC. Recovery was based on the percentage of alkaloids contained in the extract as compared to the amount added to the sand. Four replicates using 4 separate preparations of alkaloids were conducted to measure accuracy of the extraction procedure.

Quantification of Alkaloids Released into Control
and Fungal-Treated Sand by Fire Ant Workers

Effect of Inoculation on Alkaloid Release

Venom alkaloids deposited in sand by fire ants were quantified in artificial assay units with or without B. bassiana conidia. From a single laboratory colony, ants were collected randomly in groups of 50 individuals with an aspirator and placed in twenty-five 35-ml clear, plastic cups containing 10 g of sand previously treated with 250 ul of either sterile distilled water (SDW) or an aqueous suspension containing 1.2×10^9 viable B. bassiana conidia per ml (3×10^7 conidia per g sand). This rate of inoculum was chosen based on information obtained from Dr. J.L. Stimac (personal communication) and was intended to cause 70-80% mortality. Cups were then capped with plastic lids. Sand was kept moist by adding 250 ul of SDW weekly through

the cup lid with a hypodermic syringe. Holes produced by injection were sealed with silicone grease (Dow-Corning) to prevent moisture loss and escape of ants. No food was supplied during the 14-day test period to avoid disturbing the ants. Residual alkaloids in sand from 5 cups per treatment were measured at 1, 3, 8, 10, and 14 days. Ants were removed from the sand, the sand was dried at 40°C for 1 hr, and then extracted using the procedure described on page 20. Fungal- or SDW-treated sand was also checked for alkaloids prior to the addition of ants to confirm purity. Both ant mortality and infection were determined at each sample interval by surface sterilizing each cadaver with 1% sodium hypochlorite, rinsing with SDW, and incubating on water agar for 7 days at 27°C. Infection was confirmed by identifying the sporulating aerial growth of the fungus emerging from the cadaver.

Effect of Inoculation Time on Alkaloid Release

The effect of fungal inoculation time on release of alkaloids into small assay units by fire ant workers was measured using the above described protocol (page 21) with the following exceptions. Sand was autoclaved (5 hr, 120°C, 20 PSI) in order to reduce control ant mortality caused by opportunistic bacterial pathogens observed in the previous experiment (i.e., 65% control mortality). Cups containing sand were injected through the lid with 250 ul of either SDW or an aqueous suspension containing 1.93×10^7 viable conidia

per ml (4.8×10^5 conidia per g sand) either prior to or 7 days after the addition of ants. Neither the SDW nor conidial suspension were mixed into the sand. Levels of alkaloids were measured at 7, 14 and 21 days.

Following the removal of ants from the cups at each sample day, the fungistatic and insecticidal activity of sand from each treatment was measured via bioassay using A. floridanus larvae as a host. In addition to treatments containing ants, control assay units without ants, receiving identical amounts of conidial suspension or SDW were prepared and maintained. The following treatments were compared: a) inoculated ant unit sand, b) noninoculated ant unit sand, c) inoculated control sand, and d) noninoculated control sand. Five 45-day old A. floridanus (total weight = ca. 250 mg) larvae were added to each of the 4 treatment cups. Larval infection, identified by aerial mycelial growth and sporulation of the fungus on the host, was quantified after 7 days.

Fungistatic and Insecticidal Activity of Sand Venom Alkaloid-Treated Sand

Insecticidal activity of the venom alkaloids to A. floridanus larvae was measured in sand. Venom alkaloids were collected and quantified as previously described and 1 ml of the venom solutions added to 35-ml clear, plastic cups containing 10 g of autoclaved sand, yielding concentrations

of 0, 0.2, 2.0, and 4.0 ug of alkaloids per g sand. The solvent was evaporated. For each alkaloid concentration, 250 ul of either SDW or an aqueous suspension containing 2.5×10^7 viable B. bassiana conidia per ml were mixed thoroughly into the sand. Five A. floridanus larvae with an average total weight of 250 mg were then added to each cup. Both larval mortality and infection were quantified 7 days-post treatment. Infection was confirmed as described on page 20. Each treatment was replicated 3 times and the experiment was repeated twice.

Sterile and Nonsterile Sand

The fungicidal and insecticidal activities of autoclaved and nonautoclaved assay unit sand were compared according to the A. floridanus bioassay procedure on page 23 with the following exceptions. Fire ants were removed from 7-day old noninoculated cups and the sand either autoclaved (260°C, 120 PSI) or air-dried for 5 hours. The sand was then treated with either 250 ul of SDW or an aqueous suspension containing 1.93×10^7 viable conidia per ml. Each treatment was replicated 5 times.

Statistical Analyses

Data were analyzed using the linear regression and general linear models (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Mean separation analysis was conducted using Duncan's (1951) multiple range

test. Total alkaloid release into sand over time was compared between treatments using chi-square analysis.

SECTION 3 RESULTS

Effect of Fire Ant Compounds on Conidial Viability in Solid and Liquid Culture

Cuticular Hydrocarbons

Different concentrations of cuticular hydrocarbons were non-inhibitory to conidial germination of B. bassiana (AF-4). Within 24 hours, 100% germination was obtained with both solid and liquid MC media. On agarose media where hydrocarbons were the sole carbon source, conidial germination at concentrations greater than 2.52 ug of cuticular hydrocarbons per cm² ranged from 10 to 22% at 48 hours. Fungal hyphal development was minimal, and conidiation occurred at 6 days. No conidial germination occurred in liquid cultures where cuticular hydrocarbons were the sole carbon source.

Venom Alkaloids

Percent conidial germination of B. bassiana (AF-4) on MC agar after 24 hours decreased with an increase in venom alkaloid concentration (Figure 3). Germination was highly correlated ($r^2 = 0.9470$; $p = 0.0001$) with the concentration of venom alkaloids according to the quadratic relationship,

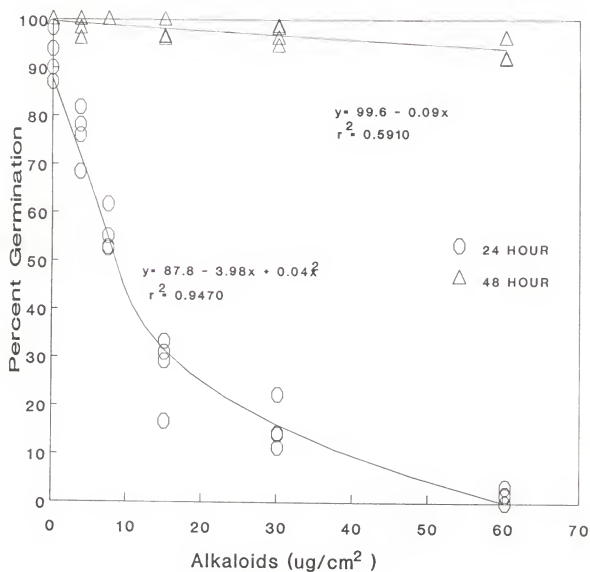


Figure 3. Percent conidial germination of *B. bassiana* on MC agar surfaces treated with different concentrations of *S. invicta* venom alkaloids at 24 and 48 hours.

$y = 87.76 - 3.98X + 0.04X^2$. At the highest concentration of alkaloids (60 ug per cm^2), less than 5% germination occurred after 24 hours. Concentrations of > 10 ug alkaloids per cm^2 caused over 50% inhibition of conidial germination.

After 48 hours, venom alkaloids had minimal effect on conidial germination with $< 10\%$ inhibition observed at any concentration, indicating a fungistatic effect on the fungus by venom alkaloids. Percent conidial germination was poorly correlated with alkaloid concentration ($r^2 = 0.5910$; $p = 0.0001$) suggesting a poor cause and effect relationship. Where venom alkaloids were applied to agarose as the sole carbon source, no conidial germination occurred.

Percent conidial germination in liquid culture, where the microtiter wells were coated with venom alkaloids, decreased with an increase in alkaloids concentration after 24 hours (Figure 4). Percent germination was highly correlated with venom alkaloid concentration ($r^2 = 0.8739$; $p = 0.0001$) according to the quadratic relationship, $y = 101.5 - 7.5X + 0.132X^2$. Alkaloid concentrations > 20 ug per cm^2 caused complete inhibition of germination at 24 hours. As in the solid media assays, percent germination increased within 48 hours, confirming the fungistatic effect of venom alkaloids on this fungal isolate. Percent conidial germination at 48 hours was highly correlated ($r^2 = 0.7025$) to concentration of alkaloids according to the quadratic relationship,

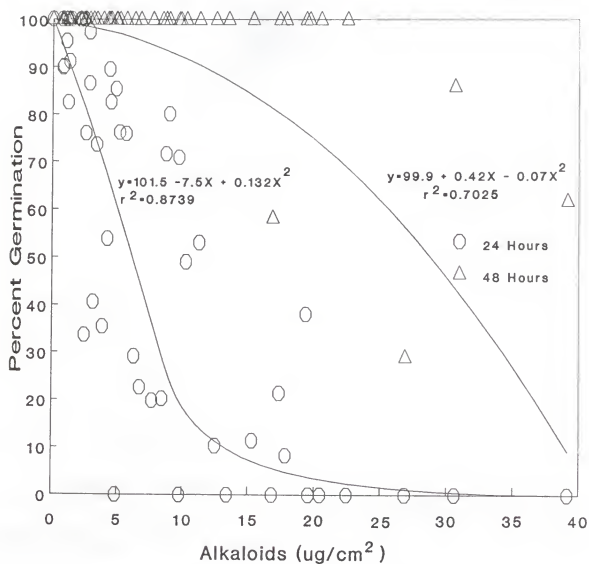


Figure 4. Percent conidial germination of *B. bassiana* in liquid culture in microtiter wells coated with different concentrations of *S. invicta* venom alkaloids after 24 and 48 hours incubation.

$y = 99.9 + 0.42X - 0.07X^2$. Concentrations < 25 ug alkaloids per cm^2 did not inhibit conidial germination at 48 hours.

Concentrations of venom alkaloids higher than 4 ug per cm^2 induced the hyphal body stage of development in *B. bassiana* in liquid culture. By comparison, conidial germination was inhibited by venom alkaloids at 24 hours (Figure 5a) while conidia in the control produced normal germ tubes (Figure 5b). At 48 hours, venom alkaloid treated conidia developed unique hyphal bodies and mycelia (Figure 6a) while conidia in the control produced vegetative mycelia (Figure 6b).

Germ tube development on venom alkaloid-treated MC agar was observed using SEM in 2 independent studies. In the first study, observation of germination on agar treated with 1.6 ug alkaloids per cm^2 revealed occasional germ tube orientation perpendicular to the agar surface after 24 hours incubation (Figure 7a). In the second study, fungal development on alkaloid-treated agar surfaces (2.5, 5, 10, 20 ug per cm^2) was not different from the control, with the germ tubes growing parallel to the agar surface (Figure 7b). No explanation can be given for this inconsistency in results, however, other workers have observed atypical germ tube orientation and fungal development in the presence of venom alkaloids (Jerry Stimac, personal comm.). Fungal colony development occurred on both alkaloid-treated and control agar surfaces within 48 hours. The hyphal body stage



Figure 5. Germination and development of *B. bassiana* in Sabouraud's dextrose broth culture in microtiter wells with a) complete inhibition of germination in *S. invicta* venom alkaloid-coated wells (20 ug per cm²) and b) normal germ tube development in control wells after 24 hours incubation at 27°C. 400x.

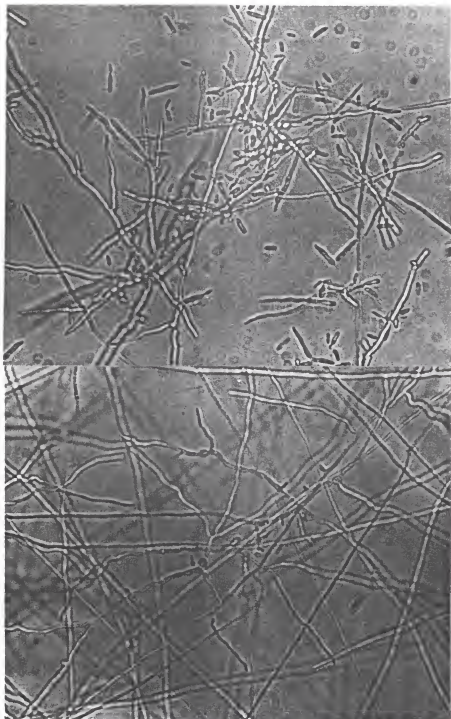


Figure 6. Germination and development of *B. bassiana* in Sabouraud's dextrose broth culture in microtiter wells with a) induction of hyphal bodies in wells coated with *S. invicta* venom alkaloids (20 ug per cm²) and b) normal mycelial growth in control wells after 48 hours incubation at 27°C. 400x.

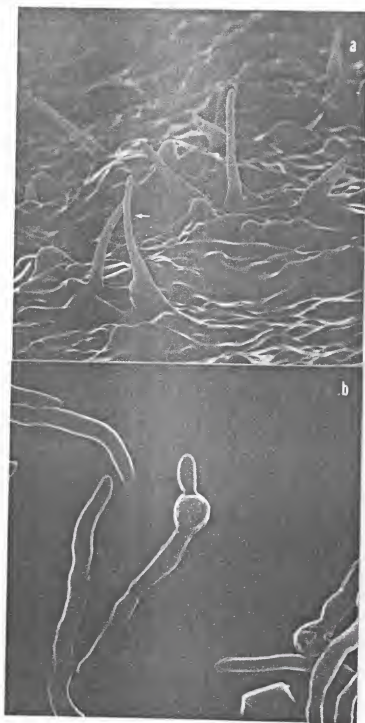


Figure 7. Scanning electron micrographs of germinating *B. bassiana* conidia on MC agar plates surface treated with *S. invicta* venom alkaloids at 27°C. Fungal development after 24 hours incubation on a) venom-treated ($1.6 \text{ ug} / \text{cm}^2$) and b) untreated agar (2,000x). Note the perpendicular orientation of germ tube in response to venom alkaloids in Figure 7a.

of development was not induced on solid agar surfaces treated with any concentration of venom alkaloids.

For B. bassiana isolates 447 and AF-4, M. anisopliae, and P. fumosoroseus, percent conidial germination decreased with increasing concentrations of venom alkaloids after 24 hours. Concentrations of alkaloids > 20 ug per cm^2 gave total inhibition of conidial germination of all fungal species and isolates (Figure 8a). Both P. fumosoroseus and M. anisopliae conidia were more sensitive to venom alkaloids than either of the B. bassiana isolates. P. fumosoroseus conidia appeared the most sensitive to venom alkaloids with complete inhibition occurring at all concentrations > 0.41 ug alkaloid per cm^2 . M. anisopliae was more sensitive than either of the B. bassiana strains, with concentrations > 10 ug alkaloid/ cm^2 causing complete inhibition. Percent conidial germination was similar for both AF-4 and 447 isolates of B. bassiana at 24 hours.

After 48 hours, the relative order of sensitivity to venom alkaloids for all fungal species was similar to the 24 hour response. P. fumoso-roseus conidia were highly sensitive to the venom alkaloids, with no germination at concentrations > 0.41 ug alkaloids per cm^2 (Figure 8b). Total inhibition of M. anisopliae occurred at a concentration of 30 ug alkaloids per cm^2 compared to 90% inhibition of the 447 isolate at that rate. B. bassiana (AF-4) was more tolerant to alkaloids than 447 with

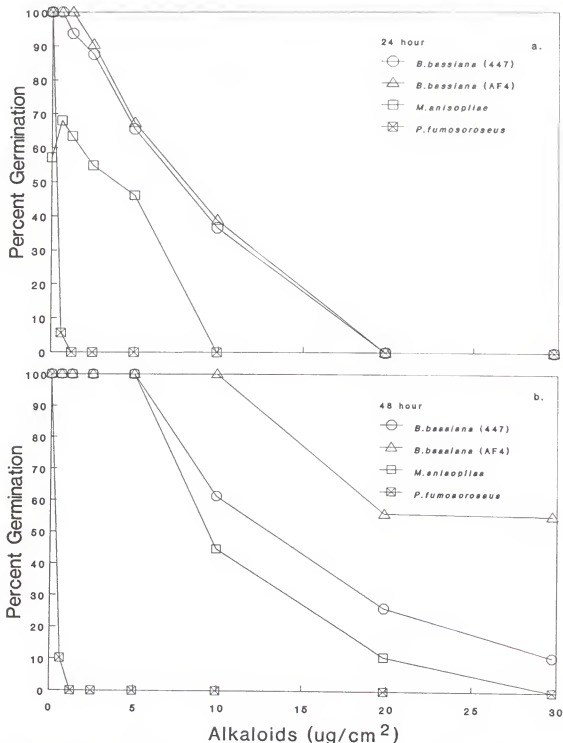


Figure 8. Comparison of percent germination of *P. fumosoroseus*, *M. anisopliae*, *B. bassiana* strain 447, and *B. bassiana* strain AF4 in liquid culture in microtiter wells coated with different concentrations of *S. invicta* venom alkaloids after a) 24 and b) 48 hours incubation at 27°C.

significantly higher ($P = 0.05$) germination at 10, 20, and 30 μg alkaloid per cm^2 . Recovery of three of the four fungal isolates from the inhibitory effects of venom alkaloids by 48 hours is consistent with the fungistatic mode of activity (Figures 3 and 4).

The hyphal body stage was observed for both AF-4 and 447 at concentrations above 5 μg per cm^2 after 48 hours. No hyphal bodies were observed in media containing M. anisopliae or P. fumosoroseus at any alkaloid concentration.

Effect of Liquid Medium on Alkaloid Activity

Coated Well Method

Inhibition of conidia in liquid culture by venom alkaloids was significantly affected by the length of elapsed time between treatment of microtiter wells with alkaloids and the addition of conidia (Figure 9). For 0 hour inoculations, total inhibition of conidial germination was observed at 24 hours for all concentrations $> 4.8 \mu\text{g}$ alkaloids per cm^2 . Complete inhibition did not occur at any alkaloid concentration for 24 and 48 hour inoculations. These data suggest that the concentration of alkaloids may be diluted by the broth in time, resulting in decreased concentration of alkaloids on the well bottom exposed to the conidia. However, germination recovery for all inoculation times at 48 hours, supports a fungistatic mode of activity for venom alkaloids against B. bassiana.

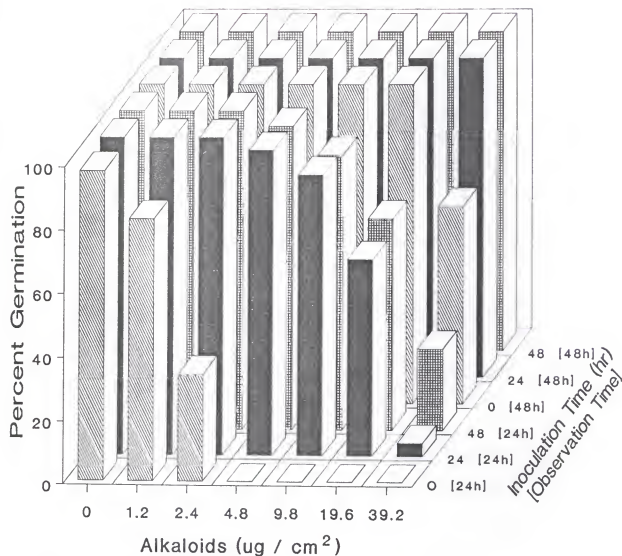


Figure 9. Residual effect in time of different concentrations of *S. invicta* venom alkaloids coated onto microtiter wells on the percent conidial germination of *B. bassiana* in liquid culture after 24 and 48 hours incubation at 27°C.

Broth Incorporation Method

Percent conidial germination after 24 hours in wells containing venom alkaloid broth solutions was completely inhibited at a concentration of 62 ng alkaloids per ul for all inoculation times (Figure 10). Percent germination increased with increasing inoculation time at concentrations ranging from 4 to 31 ng alkaloid per ul. Significant differences between the 24 and 48 hour inoculation time occurred only at the 2 and 8 ng alkaloid per ul rates. With one exception (62 ng per ul); venom alkaloids had no effect on conidial germination after 48 hours incubation confirming the fungistatic mode of activity for venom alkaloids on B. bassiana germination. Because venom alkaloids were incorporated into the broth instead of coated onto the well bottoms, dilution of the alkaloids by broth was minimized. Conclusively, increased germination at 48 hours was a fungal response and not a media dilution effect.

Quantification of Alkaloids Released Into Control and Fungal-Treated Sand By Fire Ant Workers

Alkaloid recovery efficiency in spiked sand using the methanol extraction procedure was ca. 84%. The alkaloid loss (16%) occurred during the rotary evaporation phase suggesting accumulation on the glassware. (Appendix A). Individual alkaloid components of fire ant venom were present in the venom sac preparation as 2% C₁₁ , 18.6% C_{13:0} , 15.7% C_{13:1} , 45.3% C_{15:0} , and 18.4% C_{15:1} . Relative proportions

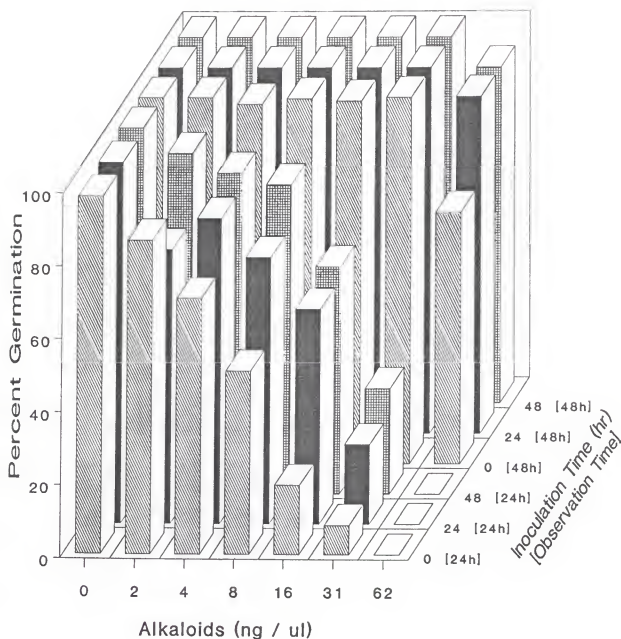


Figure 10. Residual effect in time of different concentrations of *S. invicta* venom alkaloids added to SDB on the percent conidial germination of *B. bassiana* in liquid culture after 24 and 48 hours incubation at 27°C.

of individual alkaloids in extracts of spiked sand containing all 5 venom peaks were 2.0, 16.8, 15.3, 45.7, and 20.2% respectively, indicating recovery of all peaks by the extraction process.

Effect of Inoculation on Alkaloid Release

B. bassiana conidia mixed into the sand of fire ant assay cups maintained in the laboratory resulted in higher detectable levels of venom alkaloids (Figure 11b) in time than noninoculated control sand (Figure 11a). Venom alkaloids were virtually non-detectable in both treatments between 0 and 3 days post-inoculation. Alkaloid levels peaked by day 8, with 0.8 and 0.73 ug alkaloids per g of sand being extracted from the inoculated and control sands, respectively. Cumulative alkaloid recovery from sand during the 14 day study was not significantly different (Chi-square= 4.9, df=3, P= 0.21) between control and inoculated treatments. However, alkaloid concentrations were significantly higher (P= 0.05) in inoculated sand than in control sand after 10 days. Venom alkaloid concentrations in inoculated sand were 4-fold and 2-fold higher than control sand at 10 and 14 days, respectively.

Extracts containing low concentrations of alkaloids (<2ug), contained undetectable levels of C₁₁ alkaloids. Several of these extracts also had disproportionately high C_{13:0} and C_{15:0} alkaloid levels compared to the values of MacConnell et al. (1971). GC-mass spectrophotometer analysis

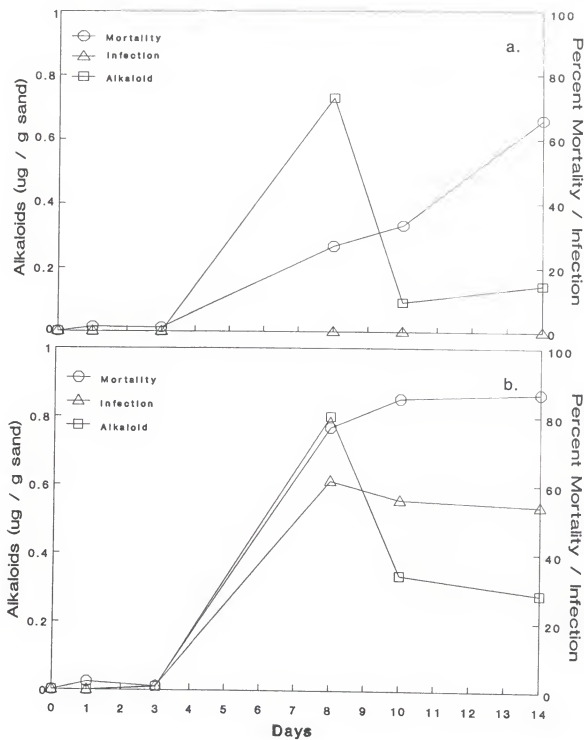


Figure 11. Kinetics of alkaloid release by *S. invicta* workers relative to mortality and infection levels of fire ant workers in a) control and b) *B. bassiana* inoculated assay unit sand (3×10^7 conidia per g sand).

conducted on those samples by Dr. R.K. Vander Meer (ARS-USDA, Gainesville, FL) indicated that venom alkaloids were present, and the increased peak size may have been caused by co-elution of non-alkaloid compounds. Since samples with disproportionately high levels of $C_{13:0}$ and $C_{15:0}$ alkaloids generally contained < 0.2 ug alkaloids per g sand, the co-eluent did not significantly effect the comparison of alkaloid recovery between treatments.

Alkaloid concentration in both control and inoculated assay unit sand declined significantly after 8 days. The mechanism responsible for this decline is unknown. No venom alkaloid breakdown products were observed by GC-MS analysis of sand extracts (Vander Meer, personal comm.). The disappearance of venom alkaloids from the nest environment should be investigated in future research on the chemical characteristics of fire ant colonies.

Ant mortality increased in both inoculated and control assay cups in time (Figure 11a-b). Mortality of fire ants in B. bassiana treated sand was nearly 60% higher ($P=0.05$) than in control sand at 8 days. Sixty percent of the fire ants in inoculated sand were infected by the fungus, which may account for the differences in percent mortality of ants between control and inoculated treatments. Both mortality and infection rates of ants in inoculated sand leveled-off after 10 days, while mortality increased to $> 65\%$ in control cups at 14 days. Opportunistic pathogenic bacteria (Serratia

marcescens and Pseudomonas aeruginosa) were present in assay units and may have contributed to the high control mortality. As a result, subsequent assays were conducted using sterile sand. Because no food was provided for the ants during the experiment in order to minimize the development of facultative microorganisms, stress in this artificial system may have also contributed to mortality toward the end of the test period.

Effect of Inoculation Time on Alkaloid Release

Venom alkaloids were detectable in both B. bassiana treated and noninoculated control assay unit sands at 7 days (Figure 12a-b). Fungal inoculation of sand prior to addition of ants (day-0) caused significant ($P= 0.07$) increases in venom alkaloid concentrations in sand compared to control assay unit sand at 7 and 14 days (Figures 12 a and b). Greater than 1 ug alkaloid per g sand was recovered from day-0 inoculated sand at 7 days compared to only 0.3 ug alkaloid per g of control sand. Likewise, 0.9 ug and 0.3 ug alkaloid per g sand was recovered from inoculated and control sands, respectively, at 14 days. Alkaloid concentration in control sand increased to 1 ug per g sand by 21 days and was not significantly different from the alkaloid concentration (0.8 ug per g sand) in inoculated sand.

B. bassiana treatment of assay unit sand at 7 days did not significantly increase the concentration of alkaloid

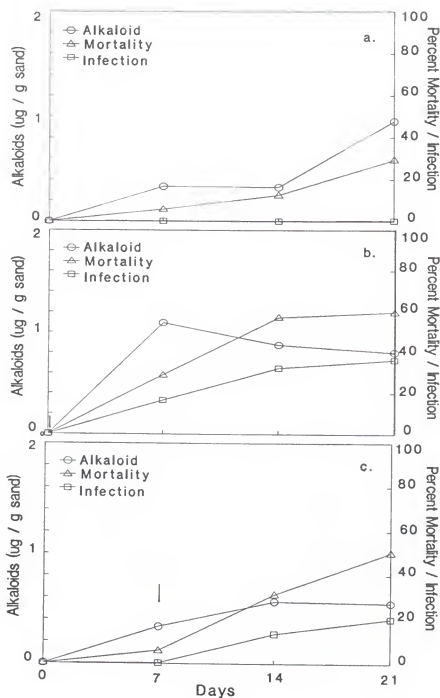


Figure 12. Recovery of *S. invicta* venom alkaloids from assay unit sand and mortality and infection rates of fire ants from a) noninoculated sand, b) sand inoculated with *B. bassiana* conidia (4.8×10^5 conidia per g sand) prior to addition of fire ants, and c) sand inoculated with *B. bassiana* 7 days post-addition of fire ants.

compared to the control at any sample time (Figures 12 a and c). Concentration of alkaloid in day-0 inoculated sand increased from 0 ug to 1 ug alkaloid per g sand within 7 days of inoculation (Figure 12b). Concentration of alkaloid in day-7 treated sand increased from 0.3 ug alkaloid prior to inoculation to only 0.6 ug alkaloid per g of sand at 7 days (Figure 12c). Differences in alkaloid concentration between the 2 treatments suggest that established ant nests are less sensitive to fungal challenge or that other factors (i.e., microbial population, toxic metabolites, etc.) in the nest decreased the sensitivity of the ants to the fungus.

In both inoculated and control assay sand, mortality increased over time (Figure 12 a-c). Mortality in the controls was < 20% at 14 days but increased to almost 30% by day 21. Mortality in inoculated sand was higher than in control sand due to infection of the ants by B. bassiana. Similar infection kinetics existed in 0-day and 7-day inoculated cups. Infection rates of 16.6% and 13.5% were observed in day-0 and day-7 inoculated sand, respectively, at 7 days post-inoculation. Infection rates were not significantly different between 0-day (32.4%) and 7-day inoculated sand (20.1%) at 14 days post-inoculation. Although alkaloid amounts were different for the 2 inoculation times, infection was similar. These results suggest that alkaloid release into sand at the levels observed here, do not affect the pathology of B. bassiana.

Mortality and infection rates of A. floridanus larvae exposed for 7 days to sand collected from the fire ant assay experiments described previously are presented in Figures 13 a-d. Mortality in the noninoculated control sand which had not been inhabited by ants previously ranged from 20 - 40% during the 21 day study (Figure 13 a) and was apparently caused by bacterial infection. Mortality in inoculated sand not inhabited with ants previously was nearly 100%, with infection rates near 80% over the 21-day study period (Figure 13 b). In noninoculated sand inhabited by fire ants previously, mortality increased from 60% in 7-day old sand to over 86% in 21-day old sand (Figure 13 c). A positive correlation ($r = 0.76301$, $p = 0.046$) existed between alkaloid content of sand and mortality in the noninoculated sand. Mortality of larvae was greater than 90% in B. bassiana inoculated fire ant assay sand (Figure 13 d). However, percent infection decreased from over 80% in inoculated non-ant sand (Figure 13 b) to less than 30% in inoculated sand from 7 to 21 day old assay units. A negative correlation ($r = 0.76521$, $p = 0.0003$) existed between sand alkaloid content and infection of A. floridanus larvae in the inoculated sand assays.

Bacterial decay of cadavers from fire ant nest sand was common in non-fungal infected larvae. Bacteria identified as Serratia marcescens and Pseudomonas aeruginosa were isolated from A. floridanus cadavers in fire ant assay sand.

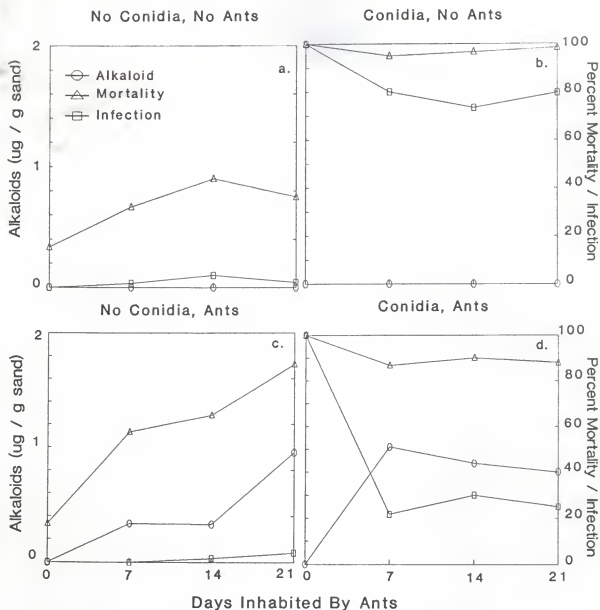


Figure 13. Percent mortality and infection of Artipus floridanus larvae after 7 days in a) sand, b) sand and B. bassiana (4.8×10^5 conidia per g sand), c) fire ant assay unit sand, d) fire ant assay unit sand and B. bassiana (4.8×10^5 conidia per g sand). Conidia were added at day 0 for both control sand and fire ant sand treatments.

Fungistatic and Insecticidal Activity of Sand

Venom Alkaloid-Treated Sand

Percent mortality of A. floridanus larvae in sand containing concentrations of venom alkaloids ranging from 0 to 4 ug per g sand was not effected by venom alkaloids (Figure 14). No significant differences in percent mortality were observed at any concentration of alkaloids tested, indicating no insecticidal activity of venom alkaloids against this insect. B. bassiana also was not inhibited by venom alkaloids added to sand. Percent infection of the larvae was 100% at all concentrations of venom alkaloids tested. The inoculum dose was identical to those applied to sand in the previously described studies. The 4 ug per g sand concentration of alkaloid was nearly 400% higher than the highest level recovered from laboratory ant assays, but below the concentrations causing delayed germination in vitro germination studies.

Sterile and Nonsterile Sand

Percent mortality and infection of A. floridanus larvae were affected by autoclaving the assay unit sand (Table 1). Percent mortality was significantly higher ($P=0.05$) in nonautoclaved sand (48%) than in autoclaved sand (20%). There was no difference in percent mortality between autoclaved (15%) and nonautoclaved (32%) control sands. All inoculated treatments had >88% mortality.

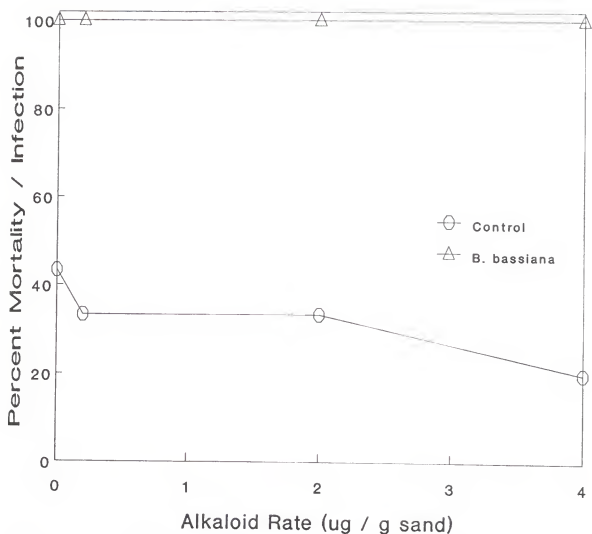


Figure 14. Effect of different concentrations of S. invicta venom alkaloids applied to sand on the survival of A. floridanus larvae and larval mycosis by B. bassiana to the larvae.

Percent infection of larvae was significantly lower in inoculated nonautoclaved sand (68%) than in inoculated autoclaved sand (100%). No differences in percent infection occurred between inoculated autoclaved (100%) and nonautoclaved (96%) control sands. No infection was observed in any of the noninoculated treatments.

Table 1. Percent mortality and infection of A. floridanus larvae in sterile and nonsterile sand inoculated with B. bassiana (4.8×10^5 conidia per g sand).

Treatment	Mortality*		Infection*	
	Sterile	Nonsterile	Sterile	Nonsterile
Sand	15.0(5.0) ^b	32.0(4.9) ^{ab}	0.0(0.0) ^b	0.0(0.0) ^b
Sand + Conidia	100.0(0.0) ^a	96.0(4.0) ^a	100.0(0.0) ^a	96.0(4.0) ^a
Assay Sand	20.0(0.0) ^b	48.0(13.6) ^a	0.0(0.0) ^b	0.0(0.0) ^b
Assay Sand + Conidia	100.0(0.0) ^a	88.0(8.0) ^a	100.0(0.0) ^a	68.0(10.2) ^b

* Means within a row followed by the same letter are not significantly different ($P=0.05$; DMRT).

SECTION 4 DISCUSSION

Effect of Fire Ant Compounds on Conidial Viability in Solid and Liquid Culture

Cuticular Hydrocarbons

Fire ant cuticular hydrocarbons were applied as a carbon source stimulated conidial germination and supported fungal growth and sporulation of B. bassiana. Boucias and Latge (1988) report similar findings for Nomuraea rileyi (Farlow) Samson with stimulation of conidial germination where host and non-host cuticular lipids were applied as carbon sources. The findings of Boucias and Latge (1988) and those of this study suggest that inhibition of entomopathogenic fungi by crude cuticular extracts (Koidsumi 1957; Evalakova and Chekhourina 1962) is due to toxic components such as short chain fatty acids (Smith and Grula 1982) and not the cuticular hydrocarbon component of the cuticle. The primary function of the hydrocarbons in and on the insect cuticle, therefore, remains one of moisture maintenance (reviewed by Hadley 1980).

Fire ant cuticular hydrocarbons consist primarily of long chain (C27) normal, monomethyl, and dimethyl branched alkanes (Lok et al. 1975; Nelson et al. 1980). Alkanes are

metabolized as a carbon source by several fungi, including M. anisopliae and N. rileyi, which utilize long chain alkanes (C_8 - C_{36}) via beta-oxidation of the terminal methyl groups (St. Leger et al. 1988a). Conidial germination occurs following a > 48 hour delay during which necessary biochemical and morphological adaptations presumably occur.

A 48 hour lag also occurs prior to B. bassiana conidial germination on fire ant cuticular hydrocarbons. It is reasonable to assume that B. bassiana also utilizes at least the normal cuticular alkanes via beta-oxidation.

Venom Alkaloids

The fungicidal activity of venom alkaloids has recently been reported (Blum 1988). In that study, individual or mixed synthetic venom alkaloids incorporated into solid medium (0.8 ng per μ l) inhibited >90% of colony growth of 8 fungal species, including 2 species, Zygorhyncus vuilleminii and Mucor sp., isolated from fire ant larvae. A Penicillium sp., also isolated from the larvae, was only slightly inhibited (<30%) by the alkaloid preparations after 53 days. In the present study, ant-derived venom alkaloid concentrations over 80-fold higher (20 μ g per cm^2 = 66.4 ng per μ l) than those used by Blum (1988) appear to be only fungistatic to B. bassiana and M. anisopliae. Only P. fumosoroseus was completely inhibited by relatively low alkaloid concentrations at 48 hours.

The increased sensitivity of P. fumosoroseus to venom alkaloids compared to B. bassiana and M. anisopliae suggests that the differences in venom alkaloid activity observed between this study and that of Blum (1988) may be due primarily to differential sensitivity of the fungal isolates to alkaloids. B. bassiana appears to be relatively tolerant to other alkaloids also. Costa and Gaugler (1989) report that the glycoalkaloids, tomatine and solanine, have only moderate effects on the growth of the fungus compared to their effects on other non-entomopathogenic fungi. The lack of inhibition of the fungus by these alkaloids, as well as by fire ant piperidine alkaloids, supports the suggestion of Costa and Gaugler (1989) that B. bassiana may have evolved detoxification mechanisms to deal with alkaloidal compounds. The delayed conidial germination response observed in B. bassiana, where venom alkaloids are applied to media, supports the suggestion of Costa and Gaugler (1989) of an induced detoxification system in this fungus. Further research should be conducted on the mechanisms involved in fungal resistance to the alkaloids.

B. bassiana is a dimorphic fungus and as such has the ability to change growth habits between mycelia and hyphal bodies (yeast-like cells) (in Aoki and Yanase 1970). Hyphal bodies normally occur as the vegetative stage of the fungus in the hemolymph of infected insects but also occur in shake-flask culture. Induction of the hyphal body stage of

dimorphic fungi is thought to be dependent upon temperature, nutrition, or a combination of temperature and nutrition (reviewed by Garraway and Evans 1984). Specifically, dibutyryl cyclic AMP added to culture media induces the hyphal body stage of Mucor racemosus. The induction is apparently due to pattern alteration of the cell wall synthesis resulting in a budding of the fungal cell instead of germ tube elongation. Hexose inhibits conversion of Mucor rouxii hyphal bodies to mycelia by blocking apical cellular growth, thus preventing cellular elongation (in Garraway and Evans 1984). In both of these fungi, dimorphic changes in growth are the results of cell wall synthesis modifications.

B. bassiana dimorphic growth also is affected by nutrition. For example, Aoki and Yanase (1970) altered the number, size, and shape of B. bassiana hyphal bodies in liquid culture by the addition of different amino acids. Thomas et al. (1987) report that developmental stages of B. bassiana in submerged culture can be altered by manipulating the amounts of phosphate and nitrate in the liquid media. Our study indicates that the hyphal body stage of this fungus can also be induced by the addition of an antagonist, specifically venom alkaloids, to liquid media. This is further supported by the induction of morphological changes, including hyphal bodies, by adding Bacillus subtilis culture extracts to B. bassiana liquid cultures (Storey and Boucias, unpublished data).

Following germination of conidia in alkaloid-treated media, there was no indication of cellular lysis of either hyphal bodies or mycelia. This suggests that the effect of the venom alkaloids on the fungus is limited to conidial germination and hyphal body initiation at germination as described above for nutrient-induced dimorphism. Similarly, venom alkaloids appear to be interfering with cell wall synthesis during germination since hyphal bodies are produced instead of elongated germ tubes. However, the exact mechanism involved cannot be deduced from the data collected in this study.

The negative chemotropic response of germ tubes to venom alkaloids observed on solid media could limit the infection process of B. bassiana by preventing cuticle penetration. Aberrant germ tube orientation on the insect cuticle following conidial germination is known to limit the infectivity of several entomopathogenic fungi (St. Leger 1988b; Wraight et al., in press). However, venom alkaloids are applied to the cuticle of larvae by fire ant workers to a concentration of only 1 ng per larva (Obin and Vander Meer 1985). This concentration is more than three logs lower than those established in vitro as fungistatic to B. bassiana and M. anisopliae and, based on in vitro assays, would not be adequate to protect the larvae from infection.

Additionally, successful infection (30-60%) of adult ants by B. bassiana in assay experiments indicates that

conidial attachment, germination, and cuticle penetration by the germ tube does occur at least occasionally or that a per os mode of infection is occurring for S. invicta workers as described for S. richteri by Broome et al. (1976).

Adult fire ant workers are liquid feeders and can filter particles, including microorganisms, as small as 1 μ m in diameter from food via fine hairs located in the infrabuccal cavity (Glancey et al. 1981). For example, spores of the microsporidian, Burenella dimorpha, ingested by adult fire ants, are filtered from liquid food and retained in the infrabuccal cavity, preventing infection of the midgut (Jouvenaz et al. 1984). Ingested B. bassiana spores (2.5 μ m x 3 μ m) should also be filtered from food by this mechanism. Per os infection of adult workers by B. bassiana would therefore most likely occur via penetration of the cuticle-lined infrabuccal cavity and not the alimentary tract. This mode of infection is currently under investigation by Mr. R. Coler and Dr. D.G. Boucias at the University of Florida.

Effect of Sand Inoculation on Alkaloid Release

Alkaloid release by fire ant workers was induced by the application of B. bassiana conidia to sand in small laboratory assay units. This supports the conclusions of Obin and Vander Meer (1985) that fire ants may use venom alkaloids as a disinfectant in the nest environment. However, the levels of alkaloid generated in nest sand were

below those established as fungistatic to either B. bassiana or M. anisopliae in vitro assays. Speculatively, other microorganisms, more sensitive to the alkaloids, could be inhibited by venom alkaloids released into soil in this manner.

Typically, host defensive reactions to fungal infection are limited to ecdysis prior to germ tube penetration of the cuticle or cellular defensive reactions in the hemolymph following entry into the hemocoel (Ferron 1981). The induction of venom release by fire ants is significant in that it represents an active external response of an insect to challenge by a pathogen. Induced defensive reactions of an insect to pathogen challenge have not previously been reported.

Induced alkaloid production has been observed in plants following foliar damage. Theoretically, this mechanism has evolved to protect plants from herbivory and pathogen attack (reviewed by Baldwin 1989). The increase in alkaloid levels in leaves is an active response triggered by herbivory and not activated by abiotic factors such as storm damage (Baldwin 1989). Likewise, venom alkaloid release by fire ant workers is an active response to challenge by heterospecifics and conspecifics (Obin and Vander Meer 1985). The data in this study indicate that pathogens are also functioning as heterospecifics in the fire ant nest and can induce alkaloidal defensive mechanisms.

The alkaloid levels reported in this study are the averages of the total alkaloids recovered from an assay unit. Areas of greatest ant activity within the nests are likely to have the highest levels of venom alkaloids. As mentioned earlier, one mechanism of venom dispersal is defensive gaster-flagging, the directional spraying of the venom at an intruder (Obin and Vander Meer 1985). Gaster-flagging behavior within the nest could result in increased levels of venom alkaloids in localized areas of the nest.

The intruder in this study was B. bassiana. It is unlikely though that venom deposition by fire ants would be directed toward the region of soil receiving the inoculum droplets because of conidial distribution characteristics of the fungus following application. Conidia applied in aqueous suspensions to soil surfaces generally penetrate into the upper 5 cm of a soil profile immediately following application (Storey and Gardner 1989). Based on this information, conidia would be distributed throughout the 1 cm deep sand of the assay cup. Fire ants were also observed tilling the sand, thereby further distributing the conidia. The dispersion of conidia throughout the sand limits the possibility of directed release and subsequent accumulation of venom alkaloids at the point of inoculation.

If venom alkaloids were applied or directed toward a small region of the nest (i.e., brood chamber), alkaloid levels observed in laboratory colonies still would not be

adequate to cause fungistasis of B. bassiana. For example, calculations of the surface area of sand based on an average particle diameter of 0.25 mm per medium sand grain (USDA Soil Texture Classification) and assuming the grain spherical for calculation purposes, indicate that 1,000 sand grains have a total surface area of 1.96 cm^2 . Maximum average venom alkaloid recovery levels (10 ug per assay cup) would provide only 5 ug alkaloid per cm^2 sand if applied to only 1,000 sand particles. Fungistasis on both solid and liquid media occurred only at concentrations $> 5 \text{ ug venom alkaloids per cm}^2$.

Biotic Factors Influencing the Survival of Insects and Fungi in Sand

Percent mortality and infection rates observed in A. floridanus larvae bioassays were influenced by a heat labile component of nest sand rather than venom alkaloids. In assay experimentation where sand was spiked with venom alkaloids, percent larval mortality was similar to that observed in nonspiked controls. Percent infection also was unaffected by venom alkaloids in sand. However, in assays comparing 7-day old sand, percent mortality of larvae was higher in nonsterile sand than in sterile sand. Alternately, percent infection was higher in sterile sand than in nonsterile sand. These data indicate that a heat-labile component(s) present in the sand is responsible for the insecticidal and

fungicidal or fungistatic activity observed in assay cup sand rather than venom alkaloids.

Nonsterile soil and soil extracts are reported as fungistatic to B. bassiana while sterile soils are not fungistatic (reviewed by Keller and Zimmermann 1989). In the present study, microbial populations were not quantified, but bacterial infections of A. floridanus larvae were often observed in sand assays. Serratia marcescens and Pseudomonas aeruginosa, as well as other unidentified bacteria, were commonly isolated from cadavers and in some cups red coloration of the sand due to S. marcescens was observed. Both of these bacteria are reported as opportunistic pathogens of fire ants (Broome 1974; Miller and Brown 1983). Apparently, these natural microbial populations were carried into the assay cups in or on the fire ant and developed in the moist sand.

The fact that B. bassiana is less infective in nonsterile sand than in sterile sand may explain the trend of lessened alkaloidal response by ants in day-7 inoculated cups than in day-0 inoculated cups (Figure 11). Day-0 inoculated sand was still sterile from autoclaving at the time of inoculation, while the microbial populations associated with the ants in day-7 inoculated sand had been proliferating for 1 week. The reduction in alkaloidal response may reflect the reduction in fungal activity due to the presence of antagonistic microorganisms.

Whether the microorganisms introduced into the assay cups are normal components of fire ant mounds or incidental to the soil environment is unknown. S. marcescens and Ps. aeruginosa have been isolated from field mounds (Brcome 1974) but they are also considered ubiquitous soil microorganisms (Freeman 1979). Many soil-inhabiting bacteria, actinomycetes, and fungi possess antimicrobial and insecticidal activity. Specific examples include Bacillus subtilis, which produces a wide range of antibiotics (Brock 1979) and the actinomycete, Streptomyces avermitilis, with pronounced insecticidal activity (Putter et al 1982). The enormity of soil microbial populations makes identification of a specific unique organism in the mound difficult without massive screening of soil organisms. However, future screening of soil for potential new antibiotics should include fire ant mound soil.

SECTION 5 CONCLUSIONS

1. Fire ant cuticular hydrocarbons were noninhibitory to B. bassiana in both solid and liquid media assays.
2. Fire ant venom alkaloids can delay germination, trigger a negative chemotropic response, and induce the hyphal body phase in B. bassiana in vitro.
3. In an artificial system, S. invicta workers will release significantly higher concentrations of venom alkaloids into when conidia are applied to a sand substrate.
4. The alkaloid concentration released by the fire ant into sand following inoculation with B. bassiana is less than levels established as fungistatic in in vitro studies.
5. Venom alkaloids showed no insecticidal activity to A. floridanus larvae nor fungistasis to B. bassiana in sand treated with fungal conidia.
6. Insecticidal and fungistatic components are present in fire ant-inhabited sand and function independently of venom alkaloids. These components were eliminated by autoclaving the sand.

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APPENDIX A
SAND EXTRACTION EFFICIENCY

Sand extraction method efficiency data.

SAMPLE	C ₁₁	C _{13:1}	C _{13:0}	C _{15:1}	C _{15:0}	ALKAL. (ug)	PER. RECOV.
Pre-Extraction							
A	3288	34348	33272	86778	38395	270.27	NA
B	4062	40224	34940	99920	41655	388.87	NA
C	3245	26322	16995	58321	19587	251	NA
Avg.	3532	33631	28402	81673	33212	290	
S.D.	375	5698	8095	17362	9726	74	
Prop.	2.0%	18.6%	15.7%	45.3%	18.4%		
Post-Extraction							
A	1787	23786	26028	68268	31553	243.09	89.94%
B	5497	27459	24386	76089	34275	298.36	76.72%
C	857	17693	12425	42821	17099	177.70	84.39%
Avg.	2714	22979	20946	62393	27642	239.72	83.69%
S.D.	2004	4028	6063	14203	7538	49.31	5.42%
Prop.	2.0%	16.8%	15.3%	45.7%	20.2%		

APPENDIX B ROTARY EVAPORATION EFFICIENCY

Venom recovery following rotary evaporation was quantified as a measure of method efficiency. Venom sacs from 5 large fire ant workers were removed and the alkaloids released into 1 ml methanol. Alkaloid concentration was determined by GC analysis with tetracosane as the internal standard. After quantification, the venom solution was added 150 ml of methanol placed in a 250 ml round bottom flask and rotary evaporated to dryness at 37°C and 25 PSI. Any remaining residue in the flask was collected in three 5 ml rinses of methanol in a 20 ml scintillation vial, evaporated to dryness under a stream of N₂, and resolubilized in 1 ml methanol. The alkaloid concentration of the rinse was quantified by GC analysis with docosane as the internal standard. Recovery following rotary evaporation was based upon the amount of alkaloid recovered compared to the amount initially added to the 250 ml flask.

APPENDIX C
EFFECT OF INOCULATION TIME DATA

Quantification of alkaloid release, ant mortality, and ant infection in control and fungal treated sand. Effect of inoculation time data.

BLOCK	TRT	TRT DAY	SAM DAY	REP	ALKALOIDS (ug/10g)	MORT	INFECT
1	0	0	7	1	2.91	.133	0
1	0	0	7	2	2.39	.018	0
1	0	0	7	3	1.48	.000	0
1	0	0	7	4	2.17	.053	0
1	B	0	7	1	1.94	.078	.041
1	B	0	7	2	9.49	.309	.115
1	B	0	7	3	11.4	.260	.156
1	B	0	7	4	19.04	.180	.017
1	0	0	14	1	5.31	.197	0
1	0	0	14	2	3.32	.350	0
1	0	0	14	3	16.97	.286	0
1	0	0	14	4	2.17	.342	0
1	B	0	14	1	14.77	.482	.167
1	B	0	14	2	2.21	.898	.296
1	B	0	14	3	12.05	.974	.370
1	B	0	14	4	26.54	.889	.500
1	B	7	14	1	1.52	.200	.043
1	B	7	14	2	1.43	.286	.021
1	B	7	14	3	5.94	.510	.019
1	B	7	14	4	1.22	.146	0
1	0	0	21	1	52.77	.373	0
1	0	0	21	2	1.7	.379	0
1	0	0	21	3	10.06	.400	0
1	0	0	21	4	11.36	.470	0

BLOCK	TRT	TRT DAY	SAM DAY	REP	ALKALOIDS (ug/10g)	MORT	INFECT
1	B	0	21	1	3.26	.793	.780
1	B	0	21	2	1.16	.980	.270
1	B	0	21	3	16.19	.605	.023
1	B	0	21	4	26.54	1.000	.020
1	B	7	21	1	6.61	.980	.373
1	B	7	21	2	4.49	.769	.237
1	B	7	21	3	3.71	.769	.039
1	B	7	21	4	3.16	.842	.158
1	B	14	21	1	2.71	.173	.0
1	B	14	21	2	2.58	.471	.050
1	B	14	21	3	33.31	.943	.029
1	B	14	21	4	12.32	.509	.0
2	0	0	7	1	2.5	.175	.0
2	0	0	7	2	9.05	.035	.021
2	0	0	7	3	4.73	.019	.0
2	0	0	7	4	2.92	.017	.0
2	B	0	7	1	29.95	.857	.774
2	B	0	7	2	3.03	.118	.060
2	B	0	7	3	6.69	.510	.180
2	B	0	7	4	33.71	.837	.455
2	0	0	14	1	1.56	.061	.000
2	0	0	14	2	3.75	.043	.000
2	0	0	14	3	1.57	.056	.020
2	0	0	14	4	2.03	.161	.000
2	B	0	14	1	14.77	1.000	.460
2	B	0	14	2	1.83	.956	.425
2	B	0	14	3	1.34	.365	.660
2	B	0	14	4	2.49	.500	.364
2	B	7	14	1	5.38	.083	.246
2	B	7	14	2	3.56	.721	.220
2	B	7	14	3	3.53	.087	.000
2	B	7	14	4	6.53	.302	.075
2	0	0	21	1	2.67	.196	.000
2	0	0	21	2	2.8	.200	.000
2	0	0	21	3	27.2	.519	.038
2	0	0	21	4	2.08	.182	.000
2	B	0	21	1	21.82	.634	.650
2	B	0	21	2	2.48	.451	.372
2	B	0	21	3	2.23	.692	.622
2	B	0	21	4	1.04	.781	.591

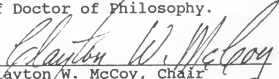
BLOCK	TRT	TRT DAY	SAM DAY	REP	ALKALOIDS (ug/10g)	MORT	INFECT
2	B	7	21	1	4.11	.414	.136
2	B	7	21	2	0.84	.143	.245
2	B	7	21	3	2.27	.148	.196
2	B	7	21	4	1.48	.279	.442
2	B	14	21	1	10.73	.458	.143
2	B	14	21	2	6.88	.905	.174
2	B	14	21	3	24.63	.314	.063
2	B	14	21	4	2.9	.268	.175
3	0	0	7	1	5.09	0.09	0.00
3	0	0	7	2	3.85	0.02	0.00
3	0	0	7	3	1.88	0.04	0.00
3	0	0	7	4	1.18	0.10	0.00
3	B	0	7	1	1.45	0.04	0.00
3	B	0	7	2	0.68	0.05	0.02
3	B	0	7	3	2.55	0.19	0.13
3	B	0	7	4	2.41	0.06	0.04
3	0	0	14	1	1.29	0.00	0.00
3	0	0	14	2	0.00	0.00	0.00
3	0	0	14	3	1.19	0.00	0.00
3	0	0	14	4	0.24	0.02	0.00
3	B	0	14	1	4.78	0.16	0.12
3	B	0	14	2	20.89	0.33	0.30
3	B	0	14	3	2.00	0.25	0.18
3	B	0	14	4	1.55	0.09	0.05
3	B	7	14	1	2.10	0.55	0.28
3	B	7	14	2	22.71	0.37	0.35
3	B	7	14	3	1.53	0.42	0.30
3	B	7	14	4	12.16	0.09	0.07
3	0	0	21	1	1.86	0.04	0.00
3	0	0	21	2	1.93	0.10	0.00
3	0	0	21	3	0.00	0.04	0.00
3	0	0	21	4	0.00	0.62	0.00
3	B	0	21	1	13.21	0.38	0.33
3	B	0	21	2	1.54	0.40	0.32
3	B	0	21	3	4.90	0.09	0.11
3	B	0	21	4	1.94	0.38	0.29
3	B	7	21	1	31.88	0.94	0.24
3	B	7	21	2	3.81	0.50	0.11
3	B	7	21	3	1.54	0.02	0.02
3	B	7	21	4	1.70	0.24	0.22

BLOCK	TRT	TRT DAY	SAM DAY	REP	ALKALOIDS (ug/10g)	MORT	INFECT
3	B	14	21	1	3.66	0.97	0.02
3	B	14	21	2	10.79	0.35	0.09
3	B	14	21	3	84.88	0.83	0.42
3	B	14	21	4	34.59	0.67	0.22

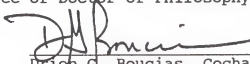
BIOGRAPHICAL SKETCH

Greggory Keith Storey was born in Griffin, Georgia, on December 10, 1961. He received his Bachelor of Science degree in applied biology from the Georgia Institute of Technology in 1984 and his Master of Science degree in entomology from the University of Georgia in 1987. Gregg continued his graduate studies in entomology at the University of Florida in August 1987 and received the degree of Doctor of Philosophy in May 1990.

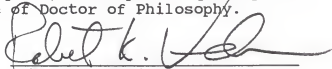
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Clayton W. McCoy, Chair
Professor of Entomology &
Nematology

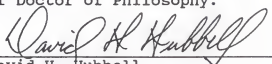
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Drion G. Boucias, Cochair
Associate Professor of
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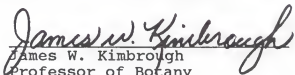
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David H. Hubbell
Professor of Soil Science

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May 1990

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